N° 10. **L. N. Ruben,** Genève. — Lucké carcinoma implants in regenerating and regressing urodele limbs.[1] (With 4 figures)

Station de Zoologie Expérimentale, Université de Genève,[2] and Reed College, Portland, Oregon, U.S.A.

Introduction

I suppose that those of us who work with Amphibian limbs would agree that one of the features of vertebrate regenerating systems that attracted us was the hope that such systems hold for eludication of the properties inherent in the phenomenon of cellular differentiation. How stabile should we consider the differentiated state of cells to be? We know for instance, that many differentiated cells possess the ability to perform at least one specialization over and above their normal adult speciality; they can become neoplastic. It would be interesting to know if they can, under unique circumstances, also become metaplastic. The question of the stability or plasticity of differentiated cells is, therefore, of interest to students of both normal and abnormal developmental processes. By virtue of its unique nature, the vertebrate limb regenerating system may someday supply us with this kind of information.

It is my purpose here to review attempts on my part to distinguish between what might be called morphological dedifferentiation and cellular or functional dedifferentiation. Because of the broad and varied usage of the term dedifferentiation in the literature, it is always necessary to define what one means by the term. By morphological dedifferentiation, I mean change in the observable

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characteristics of cells such that their organization as tissues is lost. Further, the identity of their tissue of origin is no longer discernible. Nothing is said of cellular potency. Perhaps, dissociation or dissaggregation would be appropriate terms here. The term modulation is sometimes used for a similar sequence of events in tissue culture work, but, to me at least, the term modulation seems to have implicit within it, the notion of potential constancy. Cellular or functional dedifferentiation, on the other hand, would entail a possible consequence of morphological dedifferentiation such that a change in potency would occur whereby cells would be able to gain one or more new potencies from which they were restricted during progressive differentiation, i.e. a return to a pluripotent condition followed by metaplasia or redifferentiation along a new path. The most controversial aspects of limb regeneration studies have all centered on this particular issue. The reasons for conflict are apparent when one considers that after limb amputation a blastema is established through the aggregation and proliferation of cells which have come from a variety of stump tissues. These cells which will reform the portion of the limb which has been removed all look essentially alike. Therefore, since it has not yet been possible to follow the fate of individual cells as they leave their tissue of origin and later differentiate into a tissue of the regenerate, information on this subject has been drawn largely from indirect evidence. At the moment, I favor a view on blastemal cell potency which was originally expressed by Holtzer, Avery and Holtzer (1954) and which appears to be supported by much of my own work dealing with the induction of accessory limbs in urodeles by foreign implants (Ruben, 1960, 1963). It seems likely that the cells of the limb blastema have for their primary function the production of skeletal material. If an insufficient amount of regeneration occurs, skeleton is preferentially formed. This occurs regardless of whether the blastema has been established from cells which have come from only soft mesodermal components of the limb, e.g. muscle and connective tissue, or from a cellular population which includes limb skeleton in its tissues of origin. This suggests then a limited metaplasia such that cells which come from non-skeletal mesenchymally derived tissue may form skeleton following morphological dedifferentiation. Whether former skeletal cells can form anything but

skeleton in the regenerate is not as yet known. Willis (1953), in discussing the intermutability of mesenchymal cells in pathological situations, however, has pointed out that " the reverse transformation, of osteoblast to fibroblast, is a frequent one, displayed in many familiar lesions in which the fibrous replacement of bone takes place, as in the various types of osteitus fibrosa. In these lesions, the fibrous replacement is not effected by the proliferation of pre-existing fibroblasts to fill gaps left by departed bone cells, but by a progressive transformation of bone cells into fibrocytes ". A variety of tumorous and non-tumorous pathological conditions are known which involve the more usual transformation of fibroblast to skeleton in heterotopic regions.

One aspect of the experimental approach I shall describe in a moment then, was that it represented an attempt to supply biologically marked cells to a blastema so that one might have the possibility of observing metaplasia directly.

The other aspect had to do with the issue of what effect a post-embryonic morphogenetic system might have on a cancer growing in close association with it. This approach was suggested by a hypothesis dealing with the genesis of cancer, expounded by Joseph Needham (1942) which stated that cancerous growths represent morphological escapes from weak or absent individuation fields. He defined an individuation field as a region which causes all the cells within it to form a whole; a whole organism or a whole part of an organism, e.g. an organ. His hypothesis was based upon the observation that mammals, which cannot normally regenerate lost parts, are apparently more susceptible to spontaneous and experimental cancers than are the lower vertebrates which retain the ability to regenerate. Needham's suggestion was then, that the ability to regenerate is an expression of the persistance of individuation fields in the differentiated organism and that such persistance acts as a deterant to cancer formation. He further suggested that cancer be applied to a strong individuation field in order to test whether the field could " master " the cancer and cause it to form structures normal to that field. Such regulation of a cancer might occur in two ways. First, the possibility exists that the cancer cells might be like embryonic cells in possessing an array of potentialities, which are realized only upon exposure to some strong director, the individuation field.

On the other hand, since cancer cells are probably in at least a partially differentiated state and as such may be as limited in competence as any other specialized cell type, cellular dedifferentiation to some pluripotent condition would become a prerequisite to their transformation.

Experimentation

The first regenerating system utilized in my research was that of the larval urodele limb, which I felt would supply a stronger individuation field than its adult counterpart. Two host species were used, *Amblystoma opacum* and *Amblystoma maculatum*, and the donor tissue was the renal adenocarcinoma of *Rana pipiens* which has been described in detail by Balduin Lucke (1934). One can distinguish frog donor cells from salamander host cells by their size, since the frog cells are smaller and by their differential affinity for haematoxylin; frog nuclei are more lightly stained.

The first series of my experiments tested the effect of implantation upon the cancerous epithelial structures of the implant. All implants were made subcutaneously on the dorsal forelimb surface between elbow and wrist. The results showed that over a 64 day experimental period, these epithelial elements were unaffected by the foreign environment of the *non-regenerating larval limb.*

A second series was run having the same aim as the first, however, the antihistamine, Pyribenzamine, was omitted from the operative procedure. Rose and Rose (1952) had found that the carcinoma implants would " take " in adult urodele limbs when the hosts were exposed to this antihistamine for a short period prior to implantation but that when the Pyribenzamine was omitted, the implants quickly degenerated. The antihistamine served to diminish the initial responses on the part of the host to incompatable donor tissue. It was found, that when these larvae are used as hosts, the Pyribenzamine was unnecessary, since the epithelial elements of the implants maintained their typical morphology throughout the 64 day experimental period, again in a *non-regenerating limb environment.*

The third larval series, dealing with the major problem I have outlined, entailed implanting this carcinoma, using Pyribenzamine, allowing two weeks to make sure that the implant had established

itself, amputating the limb through the donor material and looking for any morphological responses on the part of the carcinoma to the various phases of regeneration which had been slowed down by the use of low temperatures (14° C ± 2). That portion of the limb which was removed by initial amputation through the implant was used as a biopsy to study the condition of the implant tissue prior to its exposure to the regenerative processes. Regeneration of the host limbs was normal in all cases. That the implants remained unaffected by the regenerating system adjacent to it indicated that individuation field strength bears no relationship to the maintenance of the cancerous condition. In the light of this evidence, it seemed unlikely that the carcinoma cells are pluripotent in the sense of embryonic or cellularly dedifferentiated tissue. This cancerous condition then would appear to represent a differentiated state with respect to this characteristic of competence to react to individuative influences of limb morphogenesis. Assimilation was not achieved by the limb field (Ruben, 1955).

A study by Goodwin (1946) on the regeneration of various age groups of urodela had indicated that larval limbs have less completely differentiated tissues than adults and therefore are able to establish a blastema with greater facility. The level of tissue differentiation then would establish the ease or difficulty in forming a blastema, or to put it another way, the level of differentiation determines the degree of dedifferentiation (at the tissue level at least) necessary before a blastema can be established. Degree of dedifferentiation then becomes a temporal factor. It seemed possible then that an increase in the degree of dedifferentiation to which the cancer was exposed might be useful in achieving the goals of this experimentation. Since the larval experiments demonstrated the unlikelyhood that the cancer cells were themselves pluripotent, if one hoped to demonstrate any developmental effect on the cancer, it would now be necessary to bring about morphological dedifferentiation of the cancerous tissue. To test the possibility that the duration of dedifferentiation within the host limb might be a factor in determining the ability of the regenerating system to alter tissues implanted within it, the carcinoma implants were exposed to three types of experimental situations. Each situation would successively call forth a greater extension of the dedifferentiative phase of regeneration. The three situations

were as follows: 1. The regenerating system in this instance was designated as a " simple " regenerating system; it was produced in adult urodele limbs in response to a single transverse amputation performed in the usual manner at a level which passed through the donor cancer material. 2. Regeneration as it occurred under the second set of experimental conditions was designated as " exarticulate " regeneration; the method entailed amputation at the elbow, removal of the humerus after its exarticulation at the shoulder, and implantation of a piece of the cancer into the space formerly occupied by the humerus in a position just proximal to the level of amputation. The dedifferentiation phase is of greater duration in this type of system than in " simple " regenerating systems. 3. The morphological phenomenon produced in accordance with the third experimental situation involved regression as a result of denervation in one series and excessive x-radiation in another. In the first instance it was induced to occur, by amputation through the donor cancer in larval host limbs, as in the " simple " system, accompanied by complete serial denervation of the limb by resection of the brachial plexus. Larval urodele limbs will regress subsequent to amputation in the absence of peripheral innervation. These implants were exposed to a reversible regressing field; reversible in the sense that after three weekly denervations and some regression, the nerves were allowed to reenter the stump and regeneration proceeded to take place. X-irradiation of amputated larval host limbs prior to cancer implantation producing a totally regressing system was also achieved in a separate experiment.

First, the results using a " simple " regenerating system. The carcinoma implants were made into 70 adult *Triturus viridescens* hosts following the methods used in the earlier larval work. As before, the implants were placed subcutaneously on the dorsal surface of the limbs between the wrist and elbow. Pyribenzamine was used for implant protection and the amputation cleaved the implant material. The experimental period in this work was from 4 to 70 days post-amputation and the temperature was kept at 20° C. The results indicated once again that the *Rana pipiens* renal adenocarcinoma implants were refractory to the influences present in the urodele limb during " simple " regeneration (Ruben, 1956a).

Since the remaining experiments have up until now been reported only in abstract form (Ruben, 1956b, 1958) and one will be reported for the first time, I shall present them with accompanying figures.

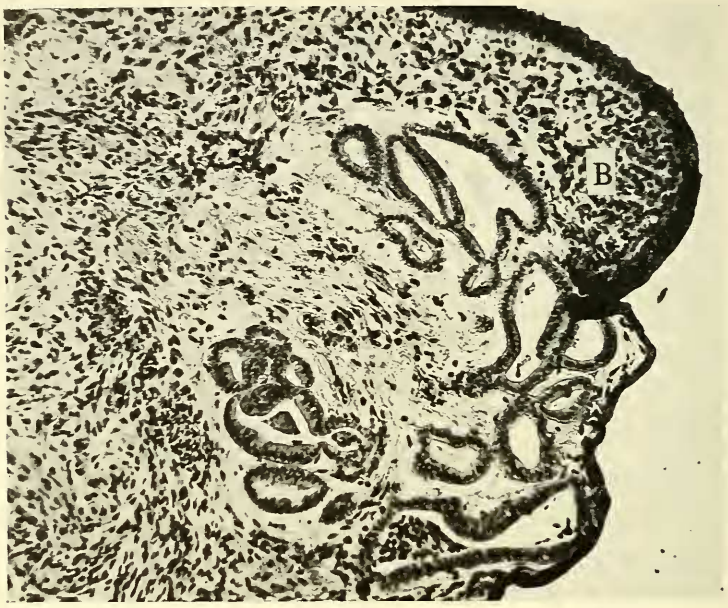

Fig. 1.
Eccentric blastema (B)
from " exarticulate " regenerate with a cancer implant
occupying a central position (28 days post-amputation).
About 80 ×

The dedifferentiation phase in " exarticulate " regenerating systems includes the post-amputation period from about 10 to 25 days at 20° C. as opposed to 7 to 13 days in " simple " regenerating systems at the same temperature. " Exarticulate " regenerating systems were established in both forelimbs of 50 *Triturus viridescens* adults. The carcinoma implants were made proximal to the level of amputation in the right forelimbs only. The left forelimbs, bearing no implants, regenerated slightly more rapidly than did the right. Nevertheless, blastemata formed in both limbs by 30 days and paddle regenerates, with digital indications

were obtained by 45 days post-amputation. It was of interest that the right forelimbs produced eccentrically placed blastemata. Figure 1 illustrates an early blastema (B) which was eccentric. Note that the cancer implant occupied the central region below the wound epithelium which quickly covered the amputation

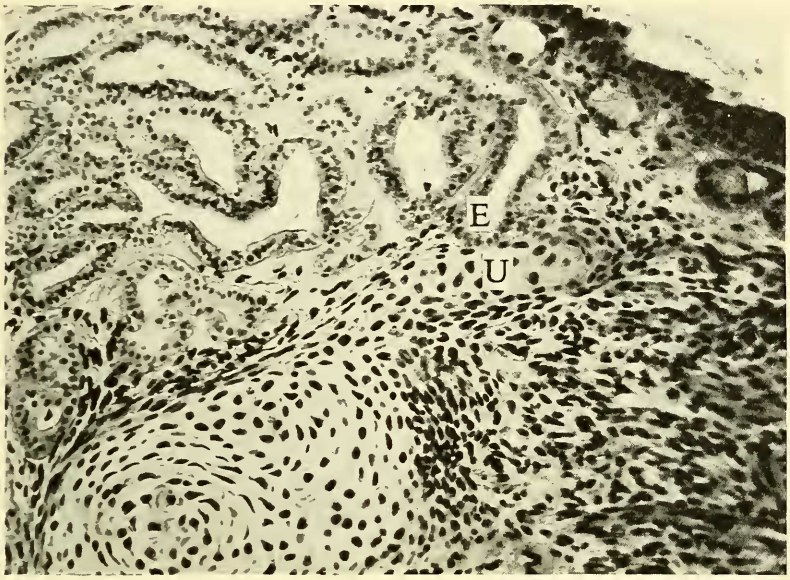

FIG. 2.
Cancer epithelium (E)
in direct contact with redifferentiating ulna (U)
(45 days post-amputation). About 120 ×

surface. As a result of THORNTON'S recent work (1960) which showed that an eccentric apical cap of the wound epithelium will establish an eccentric blastema below it, I can now by use of hindsight suggest that the centrally located implants diverted the nerves which normally enter the wound epithelium to establish the cap. This eccentric cap then may act to dissociate, morphologically dedifferentiate, if you prefer, the stump tissues below it. Following THORNTON'S lead one may further suggest that these dissociated cells then are reaggregated into an accumulation blastema below the eccentric cap. Proliferation within the reaggregated system would then lead to the type of formation

shown in Figure 1. The cancer epithelium, which had been in contact with this morphogenetic activity, remained intact and was apparently unaffected. The stability of the cancer tissue is demonstrated in a particularly striking fashion in Figure 2, which shows cancer epithelium (E) in direct contact with rediffe-

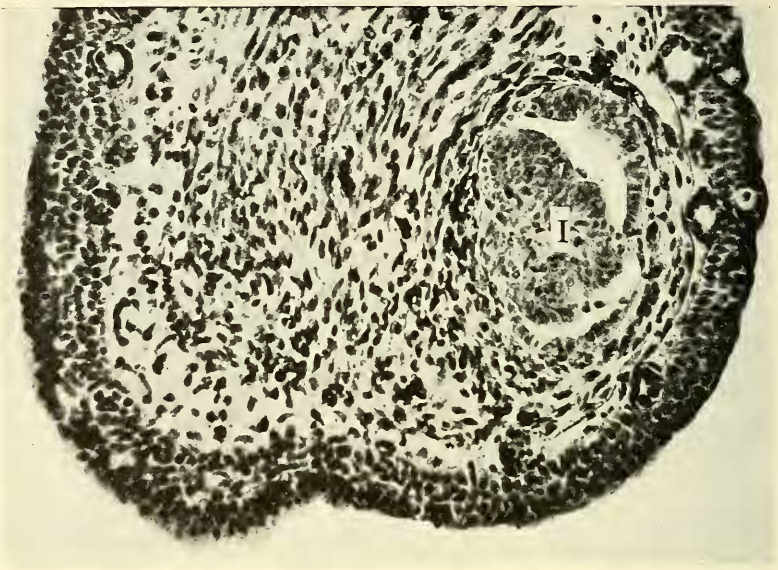

Fig. 3.
Cancer implant (I) after 10 days of regression.
The implant shows a faw mitoses and no dispersion
About 100 ×

rentiating ulna (U). Further, it seems obvious that the morphologicalogical dedifferentiation which supplied the blastemal components, which in this case had reformed humeral condyles, as well as more distal structures, failed to dissociate the tumor. No dispersed frog cells could be identified in any preparations studied. The limitations of the technique, however, are such that small numbers of individual cancer cells, which fail to seed a new population within the host tissues, might not be discerned. That skeleton-less stump can regenerate a new limb portion which is complete is, I think, further support for the idea that all mesodermally derived limb stump tissues release blastemal cells with

skeletagenous potency. The implant "take" percentage, which in this experiment was 79%, was higher than in larval or adult "simple" regenerating systems where it is usually about 65%. This effect is most likely correlated with the lack of amputation through the implant in the "exarticulate" experiments.

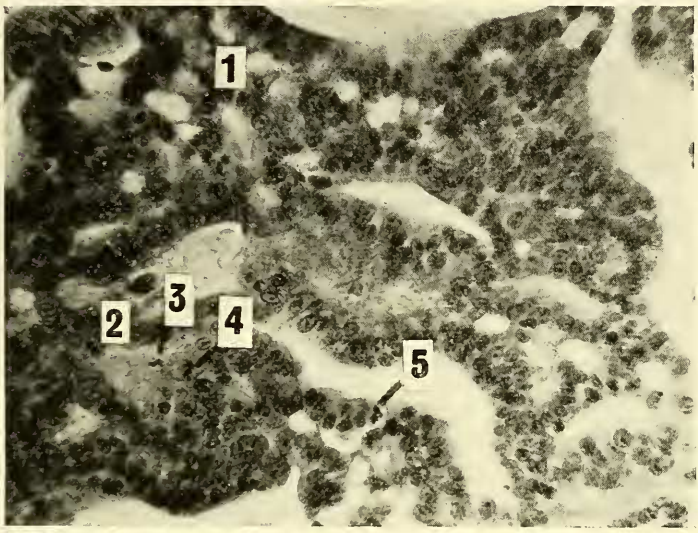

FIG. 4.
Cancer implant which had been exposed to both regression and regeneration (36 days post-amputation). Note the mitoses in the field. About 320 x

Extension of amputated host limb dedifferentiation by brachial plexus resection was produced in 162 larval *Taricha granulosa* and 50 larval *Amblystoma opacum*. The "take" percentage was substantially lower in the denervated groups than in their controls suggesting that perhaps innervation may play a role in transplantation success. Only 27% of the implants in denervated *unamputated* controls survived, as opposed to previous percentages ranging from 65 to 79 percent. Those implants which survived regression and regeneration in the experimental groups exhibited no dispersal of their cellular units and were unaltered by the reversible regression and regeneration occuring about them. Histological

examination of limbs recovered at intervals from zero to 54 days post-implantation indicated that the integrity and stability of the cancerous tissue was maintained (fig. 3). Figure 4 shows an implant which had been exposed to both regression and regeneration. Note that 5 mitotic figures are visible in the field. This mitotic activity is indicative of the " healthy " state of the cancer.

Finally, 25 larval Amblystoma opacum received 900r x-irradiation [1] on the forelimbs only and were implanted 12 hours later with unirradiated Lucké carcinoma. The remainder of the body had been protected with a ¾" lead shield. The limbs had all been amputated 6 days prior to irradiation. The implants were all made in the direction of the 6 day irradiated regenerate. X-irradiated limbs of larval urodeles will regress completely to the shoulder if they are amputated either shortly before or subsequent to irradiation. Experimental time was 50 days post-implantation. In all cases, limbs receiving the implant material regressed to a greater extent than the controls. Only one implant was recovered or identified in histological preparations of the implant bearing limbs. This implant though partially necrotic had at least some tubules in the regressing region which were intact. The lack of implant material in all of the other cases may have been due to implant degeneration or implant dissociation. The condition of the one identifiable implant and the absence of dispersed frog calls had suggested that the former was the more likely situation.

Discussion

The results of the experiments described above demonstrate that on the whole, implants of the Lucké tumor proved to be remarkably refractory to the forces at work in the immediate host environment during limb regeneration and regression. It is, of course, possible that this reluctance on the part of the Lucké tumor cells to be affected by urodele limb regeneration may be due either to the fact that they are frog in origin in a salamander host or to the foreign nature of the site, since kidney cells, whether normal or

[1] The author wishes to express his appreciation to Drs. Milton and Selma Hyman, Portland, Oregon, U.S.A. for providing their radiation facilities and their aid. The radiation factors were: Dist. = 21 cm., Time = 54 sec., KV = 220, M.A. 20, no filter, ½ value layer = 5.55 cm., and temp. = 16-18° C.

cancerous, do not usually find themselves as part of a limb system or to both. These results however, are in agreement with those of Breedis (1954) who tested an induced urodele limb sarcoma and Sheremetieva-Brunst (1955) who tested an *Axolotl* melanoma in association with urodele limb regeneration.

These failures to demonstrate positive effects on the cancers thus far tested should not be interpreted as denying the potential value of this unique test system. The use of limbs which retain morphogenetic potential for studies of these kinds provides one, I believe, with a potentially superb opportunity to observe interactions between cancer and morphogenetic processes in already differentiated organisms. Further, Breedis (1952) with his results has revealed an unusual demonstration of induction of both normal morphogenesis, in the form of supernumerary limb structures, and transplantable cancer by using the same carcinogenic agent in this system. This suggests to me that a field with morphogenetic potential may be capable or organizing cells in their early stages of response to carcinogens into structures which are quite normal for that field. Needham (1942), you will remember, had suggested that regenerative power and persistance of controlling pattern were synonomous and that such persistance acts as a deterant to cancer formation. That Breedis induced many more supernumerary limbs than cancers may be indicative of the validity of this part of Needham's argument. A review of all known occurrences of spontaneous cancers in Amphibia (Balls, 1962) indicates that no tumors of limb tissues which retain morphogenetic potential have as yet been reported. The second part of Needham's suggestion as to whether cancer in an advanced state can be so regulated, remains a controversial matter still open to test.

Summary

Experimentation involving the implantation of the renal adenocarcinoma of *Rana pipiens* into close association with a variety of situations involving urodele limb regeneration and regression is discussed with particular emphasis on the significance of the results as they apply to our knowledge of the stability of cellular differentiation and to the morphological escape hypothesis of cancer.

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